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Modulation of Relative Intrinsic Activity of Agonists at the *Alpha*-2A Adrenoceptor by Mutation of Residue 351 of G Protein $G_{i1\alpha}$

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ABSTRACT

Compared with epinephrine, the relative intrinsic activity of a series of partial agonists to activate fusion proteins between the porcine *alpha*-2A adrenoceptor and the α-subunit of G_{i1} was reduced after a single-point mutation ($Cys^{351}Gly$) in the G protein. Although UK14304 was close to a full agonist at the fusion construct containing wild-type $(Cys^{351})G_{i1\alpha}$, it was a partial agonist at that containing $Gly^{351}G_{i1\alpha}$. Moreover, although clonidine functioned as a good partial agonist to activate the fusion protein containing $Cys^{351}G_{i1\alpha}$, it was essentially an antagonist at the $Gly^{351}G_{i1\alpha}$-containing fusion protein. By contrast, incorporation of $Ile^{351}G_{i1\alpha}$ into the fusion protein resulted in all partial agonists displaying higher intrinsic activity relative to epinephrine to activate this fusion protein than the one containing the wild-type G protein sequence.

This is the first demonstration that the relative intrinsic activity of a series of agonists can be modified by a point mutation in a G protein rather than a receptor and indicates that the nature of a key contact site between a G protein and a receptor can selectively regulate partial agonist function. We provide a model for this based on the hydrophobicity of a key receptor-G protein α-subunit interaction interface.

Ligand efficacy and intrinsic activity are key concepts in pharmacology (Stephenson, 1956; Hoyer and Boddeke, 1993; Clarke and Bond, 1998). They are usually equated simply with the "strength" of an agonist ligand to transmit signal after binding to a receptor. However, a molecular understanding of the basis of these parameters would provide novel insights into the conformational alterations induced by agonist binding to G protein-coupled receptors (GPCRs) that result in G protein activation. It is particularly interesting in this regard that the relative intrinsic activity of partial agonists at the *beta*-2 adrenoceptor has been noted to be increased after mutations of this GPCR that result in degrees of agonist-independent signal transduction (Lefkowitz et al., 1993; Samama et al., 1993). Such mutations are generically described as constitutively active mutations (CAMs) (Lefkowitz et al., 1993; Samama et al., 1993).

Relative intrinsic activity can be measured at a range of points in a signaling cascade. However, due to cross-talk between pathways and varying levels of amplification throughout such cascades, differences in levels of expression of the GPCR and altered GPCR/G protein expression ratios can result in variations in this parameter when using distal points for analysis (Whaley et al., 1994; MacEwan et al., 1995). Therefore, a proximal assay point such as ligand-induced G protein activation provides a highly appropriate level for such measurements.

We constructed a series of fusion proteins between the porcine *alpha*-2A adrenoceptor and the α-subunit of the G protein G_{i1} (Wise and Milligan, 1997; Wise et al., 1997a,b; Burt et al., 1998). Because their construction defines that the ratio of expressed GPCR to G protein is always maintained at 1:1 and agonist function can be measured as activation of the GTPase activity of the G protein within the fusion construct, these have particular value in assessing relative intrinsic activity of a series of agonists (Wise et al., 1997a). We recently examined the effectiveness of a series of agonists after transient expression of an *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CAM, constitutively active mutant.

protein that was rendered insensitive to the actions of pertussis toxin by mutation of Cys^{351} of the G protein to Gly (Wise et al., 1997a). We were surprised to note that a number of *alpha*-2A adrenoceptor partial agonists, including clonidine, functioned more poorly compared with epinephrine than might have been expected. To understand the basis for these observations, we constructed further *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins in which the only difference was the nature and identity of the amino acid at residue 351 of the G protein sequence. We note that partial agonists vary in intrinsic activity relative to epinephrine with the identity of this amino acid. All partial agonists display reduced relative intrinsic activity at *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ compared with *alpha*-2A adrenoceptor wild-type $(Cys^{351})G_{i1\alpha}$, whereas they all display enhanced relative intrinsic activity to stimulate *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$. We provide a model for this based on the hydrophobicity of a key GPCR-G protein α-subunit interaction interface.

Materials and Methods

Materials. All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, Scotland). [^{3}H]RS-79948-197 (90 Ci/mmol) was purchased from Amersham International (Buckinghamshire, U.K.). [γ-^{32}P]GTP (30 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Pertussis toxin (240 μg/ml) and all other basic chemicals were purchased from Sigma (Poole, Dorset, U.K.) or Boehringer-Mannheim (Mannhein, Germany) and were of the highest purity available. Reagents for molecular biological manipulation were obtained from Promega (Madison, WI).

Construction of *Alpha*-2A Adrenoceptor-$Cys^{351}G_{i1\alpha}$ and *Alpha*-2A Adrenoceptor-$Ile^{351}G_{i1\alpha}$ Fusion Constructs. The porcine *alpha*-2A adrenoceptor (Guyer et al., 1990) was obtained from Dr. L. E. Limbird (Vanderbilt University, TN). A Cys^{351}Gly mutant of rat $G_{i1\alpha}$ was linked to the *alpha*-2A adrenoceptor as described previously to generate *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ (Wise et al., 1997a) and ligated into the *Kpn*I and *Eco*RI sites of the eukaryotic expression vector pcDNA3 (InVitrogen, San Diego, CA). Wild-type (Cys^{351}) and Ile^{351} rat $G_{i1\alpha}$ cDNAs in pcDNA3 (Bahia et al., 1998) were digested with the restriction enzymes *Sac*II and *Eco*RI. The 1.3-kb fragments so produced were recovered and ligated with *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ in pCDNA3 from which the equivalent 1.3-kb *Sac*II/*Eco*RI fragment had been removed. This resulted in generation of *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ and *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ in pcDNA3.

Cell Culture and Transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded onto 60-mm culture dishes and grown to 60 to 80% confluency (18–24 h) before transfection with pCDNA3 containing the relevant cDNA species using Lipofectamine reagent (Life Technologies, Inc.) (Wise et al., 1997b). For transfection, 2.5 to 2.8 μg of DNA was mixed with 10 μl of Lipofectamine in 0.2 ml of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 30 min before the addition of 1.8 ml of Opti-MEM. COS-7 cells were exposed to the DNA/Lipofectamine mixture for 5 h. Then, 2 ml of 20% (v/v) newborn calf serum in Dulbecco's modified Eagle's medium was added to the cells. Cells were harvested 48 h after transfection. In a number of experiments, cells were treated for the final 24 h before cell harvest with pertussis toxin (50 ng/ml).

Preparation of Membranes. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80°C after harvest as described previously (McKenzie and Milligan, 1990).

[^{3}H]RS-79948-197 Binding Studies. Binding assays were initiated by the addition of 5 μg of protein to an assay buffer (10 mM Tris·HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5) containing [^{3}H]RS-79948-197 (Wise and Milligan, 1997; Wise et al., 1997a,b) (1 nM). Nonspecific binding was determined in the presence of 100 μM idazoxan. Reactions were incubated at 30°C for 45 min, and bound ligand was separated from free ligand by vacuum filtration through GF/C filters. The filters were washed with 3 × 5 ml of assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

High-Affinity GTPase Assays. High-affinity GTPase assays were performed as described previously (Wise and Milligan, 1997; Wise et al., 1997a,b). Nonspecific GTPase was assessed by parallel assays containing 100 μM GTP. All experiments were performed at least three times on membranes prepared from individual cell transfections.

Results

We recently reported that the GTPase activity of a fusion protein between the porcine *alpha*-2A adrenoceptor and the α-subunit of a pertussis toxin-resistant (Cys^{351}Gly) mutant of the G protein G_{i1} can be stimulated by addition of the *alpha*-2 adrenoceptor agonist UK14304 (Wise et al., 1997a). To characterize agonist regulation of the enzymic properties of this fusion protein in detail, it was transiently expressed in COS-7 cells. Membranes were prepared, and the stimulation of high-affinity GTPase activity by a series of 10 *alpha*-2 adrenoceptor agonists was measured at maximally effective concentrations of each ligand (Fig. 1). The natural ligands epinephrine and norepinephrine produced the greatest levels of stimulation of high-affinity GTPase activity. UK14304 was clearly a partial agonist in comparison with epinephrine and norepinephrine (Fig. 1), as were a series of other ligands, including dexmeditomidine, BHT933, xylazine, and cloni-

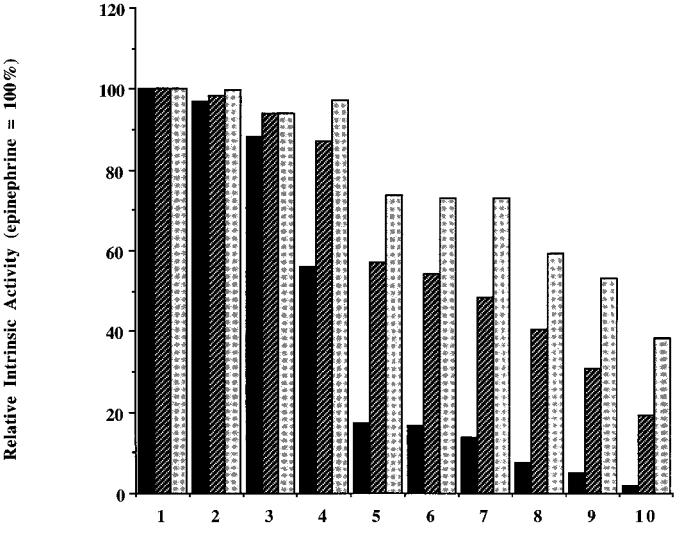

Fig. 1. Agonist relative intrinsic activity at *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins is modulated by the identity of residue 351 of the G protein. *Alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins in which residue 351 of the G protein was Gly (filled bars), Cys (hatched bars), or Ile (light gray bars) were expressed in COS-7 cells, and membranes were prepared. The capacity of maximally effective concentrations of epinephrine (1), norepinephrine (2), α-methylnorepinephrine (3), UK14304 (4), dexmeditomidine (5), BHT-933 (6), xylazine (7), clonidine (8), guanobenz (9), and oxymetazoline (10) to stimulate high-affinity GTPase activity was then recorded. Epinephrine was treated as a full agonist at each fusion protein, and activity stimulated by epinephrine (100 μM) was defined as 100%. Results are the means of three independent experiments. Errors are not displayed but in all cases were <5%.

dine. It was of interest to note that oxymetazoline, which is often described as a high-affinity partial agonist with selectivity for the *alpha*-2A adrenoceptor over the other *alpha*-2 adrenoceptor subtypes (Jasper et al., 1998) had little capacity to activate the GTPase activity of the fusion protein (Fig. 1). Other ligands, such as clonidine, also displayed substantially reduced relative intrinsic activity compared with epinephrine to values reported in the literature (Jasper et al., 1998). These values were unaffected by pertussis toxin treatment of the cells before membrane preparations (data not shown). Such results confirm that, as demonstrated previously (Wise et al., 1997a), in such transient transfections little or none of the agonist-mediated GTPase activity reflects stimulation of endogenously expressed pertussis toxin-sensitive G proteins but instead indicates activation of the fusion protein-linked G protein.

It was clearly possible that the limited effects of clonidine, oxymetazoline, and certain other ligands reflected structural constraints imposed by the nature of the fusion protein. However, because amino acid 351 of $G_{i1\alpha}$ is within the C-terminal domain known to be a key contact site between GPCRs and G proteins (Bourne, 1997; Hamm, 1998), we wanted to explore whether a single-point mutation at this position could regulate intrinsic activity in an agonist-dependent manner. A fusion protein between the porcine *alpha*-2A adrenoceptor and the α-subunit of wild-type $(Cys^{351})G_{i1}$ was thus constructed and expressed in COS-7 cells in parallel with that containing $Gly^{351}G_{i1\alpha}$. When examining the fusion protein containing the wild-type G protein, the relative intrinsic activity of all of the partial agonists was substantially greater compared with epinephrine than the values obtained at the fusion protein containing $Gly^{351}G_{i1\alpha}$ (Fig. 1). Indeed, UK14304 now displayed activity that was 90% of that of epinephrine and norepinephrine, clonidine displayed relative intrinsic activity of some 40%, and oxymetazoline was a clear partial agonist (Fig. 1).

Because we recently produced evidence in cotransfection experiments using the porcine *alpha*-2A adrenoceptor that UK14304 is able to activate $Ile^{351}G_{i1\alpha}$ to a greater extent than $Cys^{351}G_{i1\alpha}$ (Bahia et al., 1998), we also generated a fusion protein between the *alpha*-2A adrenoceptor and $Ile^{351}G_{i1\alpha}$. After expression of this fusion protein, the capacity of the same series of ligands to stimulate high-affinity GTPase activity was examined. Now, compared with epinephrine and norepinephrine, all of the ligands that functioned as partial agonists at *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ displayed higher intrinsic activity relative to epinephrine (Fig. 1) with UK14304 now acting as a full agonist.

The effects of UK14304 at each of the three fusion proteins were then analyzed in detail over a range of concentrations and compared with a maximally effective concentration of epinephrine (Fig. 2). In addition to enhanced relative intrinsic activity, as noted above, at the Ile > Cys > $Gly^{351}G_{i1\alpha}$-containing fusion proteins, the potency of UK14304 followed the same profile with EC_{50} values of $2.9 \pm 0.5 \times 10^{-8}$ M $(Ile^{351}) > 7.3 \pm 0.6 \times 10^{-8}$ M $(Cys^{351}) > 3.2 \pm 0.4 \times 10^{-7}$ M (Gly^{351}) (Fig. 2).

As an alternative means to explore variation in agonist relative intrinsic activity with alteration in residue 351 of the G protein, each of the *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$, *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$, and *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ fusion proteins expressed in membranes of COS-7 cells were stimulated by a concentration of epinephrine (100 μM) that was maximally effective at all three constructs. The capacity of varying concentrations of the *alpha*-2A adrenoceptor antagonist yohimbine or the partial agonist clonidine to modulate the effects of epinephrine was then assessed (Fig. 3). In membranes expressing *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$, yohimbine fully inhibited the effect of epinephrine in a concentration-dependent manner (Fig. 3, top). Yohimbine was also able to fully attenuate the GTPase activity of the *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ and *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ fusion proteins that had been stimulated by 100 μM epinephrine. However, the IC_{50} for yohimbine in these assays was higher at *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ $(1.4 \pm 0.2 \times 10^{-6}$ M) than at the *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ fusion protein $(3.0 \pm 0.5 \times 10^{-7}$ M) and higher again $(7.4 \pm 1.5 \times 10^{-6}$ M) at the *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ fusion protein (Fig. 3. top). Clonidine also inhibited the effects of epinephrine (Fig. 3, bottom) with IC_{50} values of (Gly) $4.8 \pm 0.5 \times 10^{-6}$ M, (Cys) $2.5 \pm 1.3 \times 10^{-5}$ M, and (Ile) $4.1 \pm 0.4 \times 10^{-5}$ M. However, much more obvious than these variations in potency for clonidine was the variation in maximal effect. At maximally effective concentrations, clonidine was able to reduce the effects of epinephrine to close to basal activity at the *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ fusion protein (Fig. 3, bottom). By contrast, maximally effective concentrations of clonidine resulted in only partial reductions of the epinephrine-stimulated GTPase activity at both the *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ and *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ fusion proteins (Fig. 3, bottom). Analysis of such experiments provided estimates for the relative intrinsic activity of clonidine compared with epi-

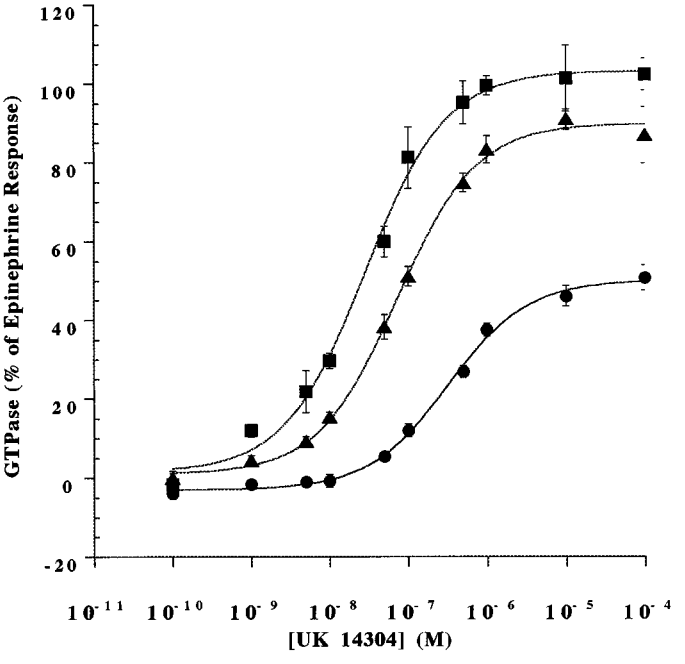

Fig. 2. The potency of UK14304 to activate *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins increases along with relative intrinsic activity. Membranes, prepared as for Fig. 1, expressing *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$ (●), *alpha*-2A adrenoceptor-$Cys^{351}G_{i1\alpha}$ (▲), or *alpha*-2A adrenoceptor-$Ile^{351}G_{i1\alpha}$ (■) were exposed to varying concentrations of UK14304 or to 100 μM epinephrine, and the stimulation of high-affinity GTPase activity was measured. Data for UK14034 are presented as a percentage of that produced by 100 μM epinephrine.

nephrine of 6 ± 2% at the *alpha*-2A adrenoceptor-Gly^{351}G$_{i1\alpha}$ fusion protein, 37 ± 7% at the *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$ fusion protein, and 64 ± 8% at the *alpha*-2A adrenoceptor-Ile^{351}G$_{i1\alpha}$ fusion protein (Fig. 3, bottom). As such, clonidine was again shown to function as a reasonable partial agonist at the fusion protein containing the wild-type G protein sequence, a better one after substitution of Cys^{351} to Ile but akin to an antagonist after the single amino acid substitution of Cys^{351} to Gly in the G protein.

The antagonist [^{3}H]RS-79948-197 displays similar affinity to bind to each of the *alpha*-2A adrenoceptor-Gly^{351}G$_{i1\alpha}$, *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$, and *alpha*-2A adrenoceptor-Ile^{351}G$_{i1\alpha}$ fusion proteins (Carr et al., 1998; and data not shown). The affinity of yohimbine to compete for the specific binding of [^{3}H]RS-79948-197 was not different among the three fusion proteins (K_i = 3.1–4.0 × 10^{-9} M) (Fig. 4, top, and Table 1). Epinephrine, clonidine, and oxymetazoline were also able to compete fully for the specific binding of [^{3}H]RS-79948-197 to the three fusion proteins (Fig. 4, bottom; and data not shown). However, although neither clonidine nor epinephrine displayed significant differences in affinity to compete for the binding of [^{3}H]RS-79948-197 to the *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$ and *alpha*-2A adrenoceptor-Ile^{351}G$_{i1\alpha}$ fusion proteins, they both displayed some 2-fold lower affinity to compete for binding to the *alpha*-2A adrenoceptor-Gly^{351}G$_{i1\alpha}$ fusion protein (Table 1).

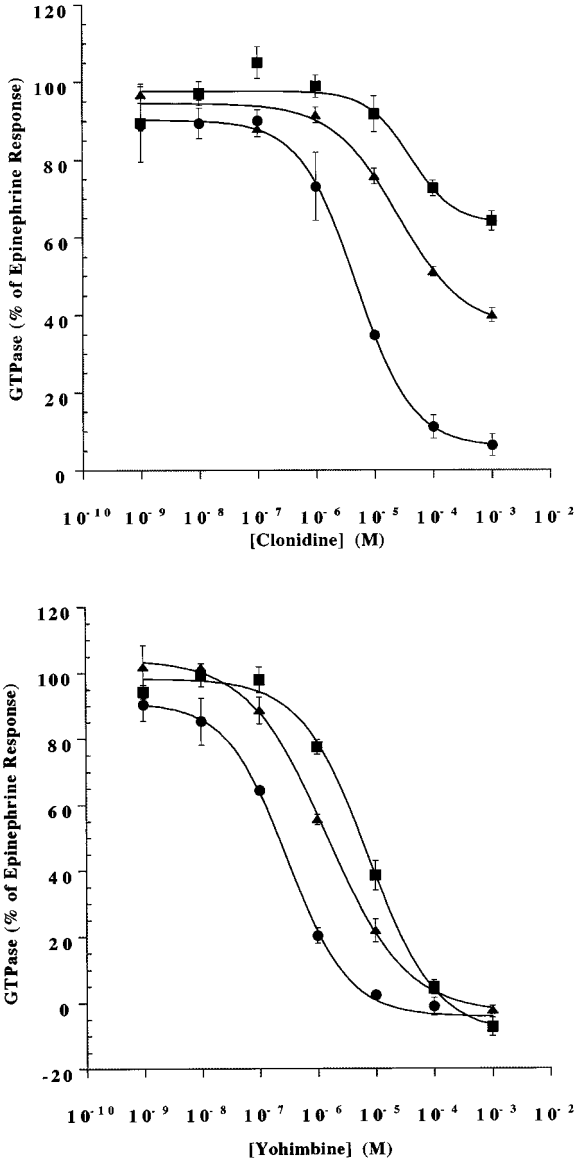

Fig. 3. The relative intrinsic activity of clonidine but not yohimbine is dependent on the identity of residue 351 of the G protein. Membranes, prepared as in Fig. 1, expressing *alpha*-2A adrenoceptor-Gly^{351}G$_{i1\alpha}$ (●), *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$ (▲), or *alpha*-2A adrenoceptor-Ile^{351}G$_{i1\alpha}$ (■) were treated with combinations of a maximally effective concentration of epinephrine (100 μM) and varying concentrations of either yohimbine (top) or clonidine (bottom).

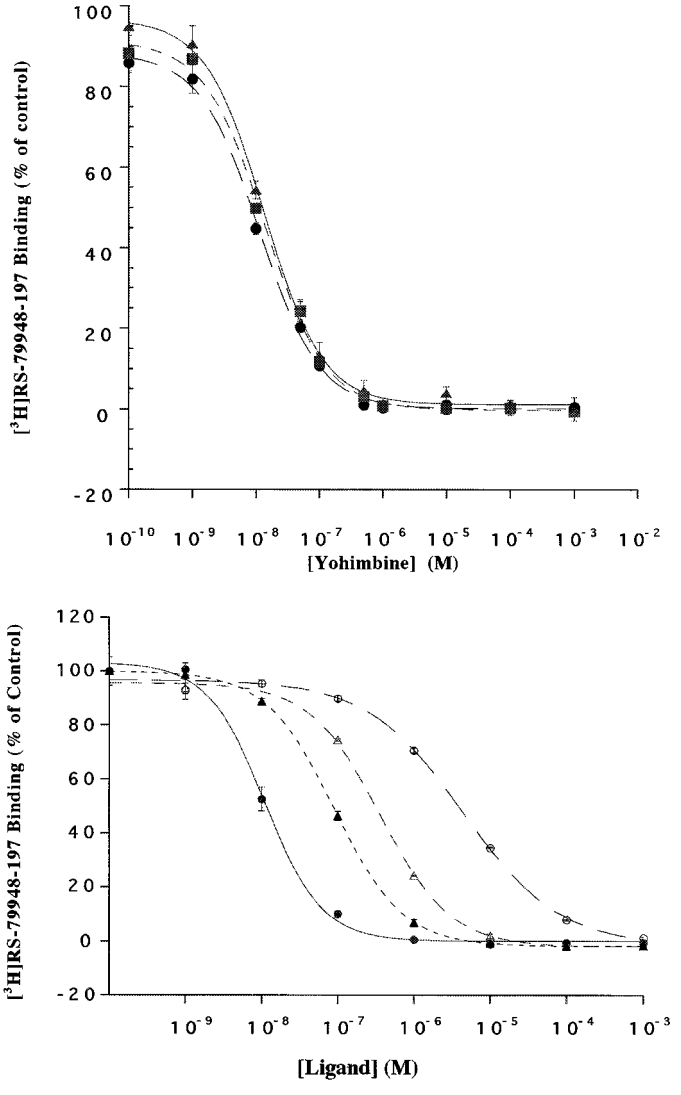

Fig. 4. The capacity of agonists and antagonists to compete with [^{3}H]RS-79948-197 for binding to *alpha*-2A adrenoceptor-G$_{i1\alpha}$ fusion proteins. Top, competition binding experiments were performed between [^{3}H]RS-79948-197 (1 nM) and varying concentrations of yohimbine in membranes prepared after transfection of COS-7 to express *alpha*-2A adrenoceptor-Gly^{351}G$_{i1\alpha}$ (●), *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$ (▲), or *alpha*-2A adrenoceptor-Ile^{351}G$_{i1\alpha}$ (■). Bottom, the capacity of yohimbine (●), oxymetazoline (▲), clonidine (△), and epinephrine (○) to compete with [^{3}H]RS-79948-197 (1 nM) for binding to *alpha*-2A adrenoceptor-Cys^{351}G$_{i1\alpha}$ in membranes of COS-7 cells was assessed.

Discussion

The basis of relative agonist intrinsic activity is a major practical as well as theoretical issue in pharmacological studies (Stephenson, 1956; Hoyer and Boddeke, 1993; Clarke and Bond, 1998). Although generally considered as the "strength" of the agonist to generate and promote a signal, in molecular terms it must reflect the capacity of the ligand to stabilize a conformation of the GPCR able to activate a cognate G protein. Considerable interest has been accorded observations of mutations in GPCRs that result in receptor activity in the absence of agonist ligands (Lefkowitz et al., 1993; Samama et al., 1993). So-called CAM GPCRs have been considered to be likely to provide insights into conformations of wild-type receptors that are stabilized on addition of agonist ligands. Interestingly, given the results of this study, a regularly observed feature of a number of CAM GPCRs has been the enhanced relative intrinsic activity of partial agonist ligands compared with their effects at the wild-type GPCR (Lefkowitz et al., 1993; Samama et al., 1993).

If the relative intrinsic activity of agonists to transmit a signal from GPCR to G protein can be modified by structural alterations in the GPCR, it should be inherently true that modifications in G protein structure should also alter this parameter. Moreover, this might be anticipated to be particularly obvious if such alterations in the G protein were located at key contact sites with GPCRs. Although certain mutations in G proteins are known to prevent guanine nucleotide exchange and/or hydrolysis (Bourne, 1997), these would not be anticipated to result in more subtle modifications of relative intrinsic activity of different agonists.

The details of interfacial surfaces responsible for productive GPCR-G protein interactions have yet to be fully mapped. However, the extreme C-terminal domain of G protein α-subunits certainly is a key contact site (Bourne, 1997; Hamm, 1998). In the G_i family of G proteins, a conserved Cys residue 4 amino acids from the C terminus acts as the acceptor for ADP-ribose transferred catalytically from NAD^+ by pertussis toxin. This modification essentially attenuates productive interactions between receptors and this family of G proteins. Members of the G_i family are routinely coexpressed, and therefore a null background is lacking for expression and functional studies. As such, a number of groups have generated mutations in G_i family members in which this Cys residue has been converted to either Ser or Gly to render the proteins insensitive to the actions of pertussis toxin (Hunt et al., 1994; Senogles, 1994; Chuprun et al., 1997; Wise et al., 1997c; Yamaguchi et al., 1997). Such mutant proteins have been of great use, but little attention has been paid to the effects these mutations may have on the detailed pharmacology of signal transduction. Recently, we converted Cys^{351} of $G_{i1\alpha}$ into every possible amino acid. After individual coexpression of each of these with the porcine *alpha*-2A adrenoceptor, we demonstrated a spectrum in the capacity of the agonist UK14304 to stimulate binding of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate to these mutants (Bahia et al., 1998).

We have also been developing the use of fusion proteins between GPCRs and G protein α-subunits to examine the details of interactions between specific pairs of signaling polypeptides (Wise and Milligan, 1997; Wise et al., 1997a,b; Burt et al., 1998). This strategy provides a wealth of useful features, including the knowledge that the expression ratio of GPCR and G protein must be 1:1, the necessity of proximity of the protein partners after expression, and, most important for the current study, the capacity to consider and analyze the construct as an agonist-activated GTPase on which detailed enzyme kinetics and pharmacology can be performed.

The first construct we generated was between the porcine *alpha*-2A adrenoceptor and a pertussis toxin-insensitive Cys^{351}Gly mutant of $G_{i1\alpha}$ (Wise et al., 1997b). This produced excellent responses to both epinephrine and UK14304, which were used to provide direct turnover numbers for agonist-induced guanine nucleotide hydrolysis. However, we noted that oxymetazoline, which is routinely described as a high-affinity and *alpha*-2A adrenoceptor-selective partial agonist (Jasper et al., 1998), failed to cause any significant stimulation of the high-affinity GTPase activity of the fusion construct (Fig. 1). Furthermore, certain other agonists displayed substantially weaker intrinsic activity compared with epinephrine than routinely reported in the literature (Fig. 1). These observations could be viewed as evidence that the fusion protein approach was flawed and not useful for detailed pharmacological analysis. However, we selected to explore a more interesting possibility (i.e., that the Cys^{351}Gly mutation in $G_{i1\alpha}$ selectively limited its activation in a manner that was dependent on the intrinsic activity of the ligand). Expression of fusion proteins between the porcine *alpha*-2A adrenoceptor and wild-type $(Cys^{351})G_{i1\alpha}$ and $Ile^{351}G_{i1\alpha}$ confirmed this concept (Fig. 1). For the fusion protein containing wild-type G protein sequence, the relative intrinsic activity compared with epinephrine was now increased for all the partial agonists at *alpha*-2A adrenoceptor-$Gly^{351}G_{i1\alpha}$. Importantly, their relative intrinsic activity was further increased when we examined a fusion protein containing $Ile^{351}G_{i1\alpha}$ (Figs. 1 and 2).

To explore this in greater detail, the capacity of clonidine to compete with epinephrine for stimulation of high-affinity GTPase activity was compared at each construct. As a control, we demonstrated that maximally effective concentrations of the *alpha*-2 adrenoceptor antagonist yohimbine would fully compete with epinephrine (Fig. 3, top). It was noted, however, that although the binding affinity of yohimbine was identical at the three fusion proteins, as might be anticipated for an antagonist, the potency of this ligand to compete for epinephrine-stimulated GTPase activity was not. Yohimbine potency increased in the order $Gly^{351} > Cys^{351} > Ile^{351}$ (Fig. 3, top). This was not inherently surprising because the potency of UK14304 to stimulate the GTPase ac-

TABLE 1
Affinity of *alpha*-2 adrenoceptor ligands at *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins: Effects of the identity of residue 351 of the G protein

Residue 351 of $G_{i1\alpha}$	Corrected IC_{50} Values		
	Yohimbine	Clonidine	Epinephrine
		nM	
Gly	3.8 ± 1.1	104 ± 17	2040 ± 550
Cys	4.1 ± 1.2	66 ± 5	1210 ± 420
Ile	3.0 ± 0.9	62 ± 17	690 ± 210

Competition binding studies between [^{3}H]RS-79948-197 (1 nM) and varying concentrations of yohimbine, clonidine, and epinephrine for the binding site of *alpha*-2A adrenoceptor-$G_{i1\alpha}$ fusion proteins containing either Gly, Cys, or Ile at residue 351 of the G protein were performed. IC_{50} values derived from such competition experiments were subsequently corrected for receptor occupancy based on a measured K_d for [^{3}H]RS-79948-197 of 0.4 nM (Wise et al., 1997a). Data are derived from at least three independent experiments.

tivity of the three fusion proteins also varied but in the reverse order (Fig. 2). It is also noteworthy that in cotransfection experiments with the porcine *alpha*-2A adrenoceptor and residue 351 mutants of $G_{i1\alpha}$, the potency of UK14304 increased, as did the maximal capacity of the individual forms of $G_{i1\alpha}$ to be stimulated (Bahia et al., 1998).

Clonidine was able to compete with epinephrine for stimulation of high-affinity GTPase activity at each of the three fusion constructs (Fig. 3, bottom). However, at maximally effective concentrations of clonidine, the results were very different. At the *alpha*-2A adrenoceptor-Cys^{351}$G_{i1\alpha}$ fusion protein, stimulated GTPase activity was reduced to some 40% of that produced by epinephrine. This value was in good accord with that obtained from the direct addition of clonidine (Fig. 1). This is a reflection that as clonidine competes with epinephrine to fill the ligand-binding site of the fusion protein population, the asymptote reached when clonidine has fully displaced epinephrine must reflect the relative intrinsic activity of clonidine compared with epinephrine. However, this value was distinctly different at the *alpha*-2A adrenoceptor-Gly^{351}$G_{i1\alpha}$ fusion protein. At maximally effective concentrations, clonidine was almost as effective as yohimbine in suppressing epinephrine-stimulated GTPase activity, with an estimated relative intrinsic activity of only 7%. Most interestingly, when using the *alpha*-2A adrenoceptor-Ile^{351}$G_{i1\alpha}$ fusion protein, clonidine displayed a relative intrinsic activity of some 60% (Fig. 3, bottom). Confirmation that these results were not related simply to a low affinity of clonidine to occupy the binding site of the *alpha*-2A adrenoceptor-Gly^{351}$G_{i1\alpha}$ fusion protein compared with those containing Cys^{351}$G_{i1\alpha}$ or Ile^{351}$G_{i1\alpha}$ was produced in classical [^{3}H] ligand-binding studies. The *alpha-2* adrenoceptor antagonist [^{3}H]RS-79948-197 displayed equal affinity to bind to each fusion construct. Clondine and epinephrine were each able to fully compete with the antagonist for binding at each fusion protein construct, and the higher affinity of clonidine compared with epinephrine for this site (Fig. 4, bottom) ensured that clonidine would compete effectively with epinephrine in the GTPase inhibition studies (Fig. 3, bottom).

The capacity of UK14304 to stimulate binding of [^{35}S]GTPγS to residue 351 mutants of $G_{i1\alpha}$ is highly correlated with the hydrophobicity of the amino acid at this position (Bahia et al., 1998). Furthermore, hydrophobic Leu residues are found invariantly throughout the family of mammalian G protein α-subunit at positions -3 and $+2$ in relation to this site. An obvious hypothesis is that these interactions would benefit from additional hydrophobicity at residue 351. An interpretation of the effects noted herein is that "strong" full agonists can overcome a suboptimal GPCR-G protein interface in this region to produce sufficient stabilization and interaction to allow effective G protein activation. By contrast, "weak" partial agonists are even more ineffective in attempting to promote G protein activation when this GPCR-G protein interface is made less hydrophobic in character.

These results offer a conceptually simple, but highly attractive, picture of how agonist relative intrinsic activity is manifest after binding to the *alpha*-2A adrenoceptor to allow activation of the G protein $G_{i1\alpha}$ and how it can be modulated. Hydrophobic interfaces are clear candidates for determining the effectiveness of protein-protein interactions. It will be of considerable interest to analyze whether this is true for activation of $G_{i1\alpha}$ by other GPCRs and, indeed, for activation of other G proteins by the *alpha*-2A adrenoceptor. It is noteworthy in this regard that a 4-amino acid motif of the M2 muscarinic acetylcholine receptor, which is distinctly hydrophobic (Val^{385}, Thr^{386}, Ileu^{389}, and Leu^{390}) and which is predicted to lie on one face of an α helix close to the interface of the third intracellular loop and sixth transmembrane region, has been demonstrated to have selective capacity to interact with the C-terminal portion of G_i family G proteins (Liu et al., 1995). Related Val- and Leu-rich sequences are commonly found at the water-lipid interface at the end of the predicted third intracellular loop and sixth transmembrane element of many G_i-linked GPCRs. Furthermore, the Thr residue of this motif is particularly conserved in GPCRs with related function. Mutation of this Thr in the human *alpha*-2A adrenoceptor has been reported to cause constitutive activity (Ren et al., 1993). Based on the results provided herein, mutational analysis of this region of the *alpha*-2A adrenoceptor might also be predicted to provide further novel insights into the basis of agonist-relative intrinsic activity and the selectivity of GPCR-G protein interactions.

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